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Reassessment of the putative chaperone function of prolyl-*cis/trans*-isomerases

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Abstract

The folding of proteins can be assisted by two unrelated groups of helper molecules. Chaperones suppress non-productive side reactions by stoichiometric binding to folding intermediates, and folding enzymes catalyze slow rate-limiting steps of folding. We reinvestigated, whether peptidyl-prolyl-*cis/trans*-isomerases of the cyclophilin type act simultaneously as chaperones and as folding catalysts in the reactivation of human carbonic anhydrase II, as reported recently [Freskgård, P.-O. et al. (1992) Science 258, 466–468; Rinfret, A. et al. (1994) Biochemistry 33, 1668–1673]. No increase in the yield of native carbonic anhydrase-II could be detected in the presence of three different prolyl isomerases, when reactivation was followed by a sensitive assay for an extended time of 4 h. We conclude that the role of prolyl isomerases in the refolding of carbonic anhydrase can be explained solely by their isomerase activity. There is no need to invoke simultaneous functions as chaperones for these folding catalysts.

Key words: Peptidyl-prolyl-*cis/trans*-isomerase; Carbonic anhydrase; Chaperone; Folding

1. Introduction

The native structure of proteins is determined by their amino acid sequence, and folding to the native state is a spontaneous process [1]. Protein folding in vivo and in vitro can be assisted by two types of unrelated helper proteins [2,3,4]. Chaperones often increase the yield of native protein in folding experiments. They bind to incompletely folded poly-peptides and thus prevent aggregation, which competes with productive folding. The binding to chaperones can also maintain precursor proteins in a transport competent form [5]. Generally chaperones act stoichiometrically, they increase the yield of native molecules, and usually do not accelerate folding. The other group of folding helpers are enzymes such as the peptidyl-prolyl-*cis/trans*-isomerases (PPIases; EC 5.2.1.8) and the protein disulfide isomerases (PDI; EC 5.3.4.1). They catalyze slow protein folding steps and thereby accelerate folding. In contrast to the chaperones, these enzymes are active in catalytic amounts. Experiments in vivo suggest that the enzymatic activities of PDI and PPIases are involved in cellular folding reactions [6,7]. An increase in the yield of correctly folded protein due to the catalytic action of these enzymes can be expected when correct folding is kinetically favoured over non-productive side reactions, such as aggregation or digestion [8].

Recently, both an acceleration of folding and an increase in the yield of native protein was observed for human carbonic anhydrase II (HCAII) when a prolyl isomerase of the cyclophilin type was present during refolding, and it was concluded that cyclophilins possess simultaneously a prolyl isomerase activity and a chaperone function in protein folding [9,10]. A further elucidation of this presumed dual function of cyclophilins is of central importance for understanding the mechanisms of assisted protein folding reactions. Therefore we reinvestigated the folding of HCAII in the presence of PPIases. We used an improved method to assay the enzymatic activity of HCAII during refolding and we followed reactivation in the absence and presence of two different cyclophilins over an extended period of time. In addition, cytosolic recombinant human FKBP (rhFKBP12cy), a representative of the family of the FK506 binding PPIases, was included in the experiments to examine whether these proteins can also function as chaperones. We find two slow phases in the refolding of HCAII, both of which are catalyzed by all three PPIases. After one hour the extent of reactivation is indeed higher in the presence of prolyl isomerases as observed previously [9,11]. Uncatalyzed reactivation is, however, not bcomplete within 1 h, and after 4 h the same final yields of reactivation are observed in the presence and absence of PPIases. We conclude from these results that the role of these PPIases in the folding of HCAII can be explained solely by their isomerase activity.

2. Materials and methods

Guanidinium chloride (GdmCl) and Tris-buffer, ultra pure, were purchased from Schwarz Mann (Orangeburg, NY). Human carbonic anhydrase II (HCAII) was kindly provided by Dr. Uno Carlsson (Linköping University, Sweden). The molecular mass was determined to be 29097 ± 4 by mass spectrometry and the N-terminal sequence was AHWGYGKHN. RhCyp18cy and rhFKBP12cy were a kind gift from

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Dr. Kurt Lang, Boehringer Mannheim. Purification of porcine kidney Cyp18cy is described in [12].

2.1. Denaturation and renaturation

HCAII (17 μM) was denatured in 5.0 M GdmCl, 0.1 M Tris/H_2SO_4, pH 7.5 for 1 h at 20°C. Reactivation was started by a rapid 17-fold dilution with 0.1 M Tris/H_2SO_4, pH 7.5, 20°C to a final HCAII concentration of 1 μM. Reactivation kinetics and yields were determined following the esterase activity of HCAII towards 4-nitrophenyl-acetate (4-NPA) by a continuous as well as by a discontinuous assay method.

2.2. Continuous reactivation assay

Reactivation of HCAII was initiated by rapid 17-fold dilution of 17 μM denatured HCAII with 0.1 M Tris/SO_4, pH 7.5 containing 0.2 mM 4-NPA at 20°C. Cleavage of 4-NPA by HCAII was followed by the change in absorbance at 348 nm (A_{348} is the isosbestic point for 4-nitrophenol/4-nitrophenolate) in a Uvicon 940 spectrophotometer, using thermostated 1 cm cuvettes. Because of the limited solubility of the substrate 4-NPA in aqueous solution, its concentration in the assay is at least one order of magnitude smaller than its K_m-value [13]. Under this condition two processes occur during reactivation. The substrate concentration [S] decreases according to a first order reaction while the amount of native catalytically active enzyme increases over reactivation time. Therefore the slope of a plot of A_{348} versus reactivation time is not directly proportional to the amount of active enzyme. To account for the decrease in [S] during the reactivation process, a linearization according to a first-order reaction (a plot of $\ln([S_o]/([S_o]-[P]))$ versus reactivation time t) is applied. The slope of this plot is proportional to the amount of active enzyme [E] that is present at the respective reactivation time. A plot of these slopes as a function of the time of reactivation ($d(\ln([S_o]/[S_o]-[P]))/dt$) versus t) thus reflects the fractional increase of native protein in the course of refolding. $[S_o]$ is given by the A_{348} value of the respective sample in which all substrate molecules are hydrolyzed. For the native control, a constant slope is observed over time in such a plot since the concentration of active enzyme does not change during the assay. Its ordinate value was set to 100% native enzyme.

2.3. Discontinuous reactivation assay

Reactivation of HCAII was initiated in the absence of substrate. To determine the amount of reactivated HCAII, 100 μl aliquots were withdrawn from the reactivation mixture after various times and after completion of reactivation (>180 min) and added to 700 μl 0.1 M Tris/SO_4, pH 7.5, to give a final concentration of 0.5 mM 4-NPA, 0.0375 M GdmCl 1 mM EDTA and 0–0.125 μM active enzyme. Under these conditions, [S] is approximately constant during the time required for the analysis (30 s), so that the initial slope of a plot of A_{348} versus time (v_o) is proportional to [E]. The amount of reactivated enzyme was determined from v_o relative to the native control and plotted as a function of reactivation time.

3. Results and discussion

3.1. Methods to follow the reactivation of HCAII

During the refolding of HCAII a native-like CD spectrum in the amide region and a native-like fluorescence emission are regained within the deadtime of manual mixing (<15 s). These two probes have been used to follow the rapid formation of an intermediate of the molten globule type at the beginning of the folding process of the bovine enzyme [14]. This fast reaction is followed by several slow processes, which involve prolyl isomerizations. They determine the overall rate of folding and are required for the regain of the catalytic activity of HCAII. The proline-limited slow steps of folding can therefore be followed by monitoring the catalytic activity as a function of the refolding time. HCAII shows two activities, which can be used to assay its enzymatic function: the hydration of carbon dioxide and the hydrolysis of esters. In both reactions a water molecule is added to the carbonyl atom of the substrate, which is catalyzed by the same active site [13,15].

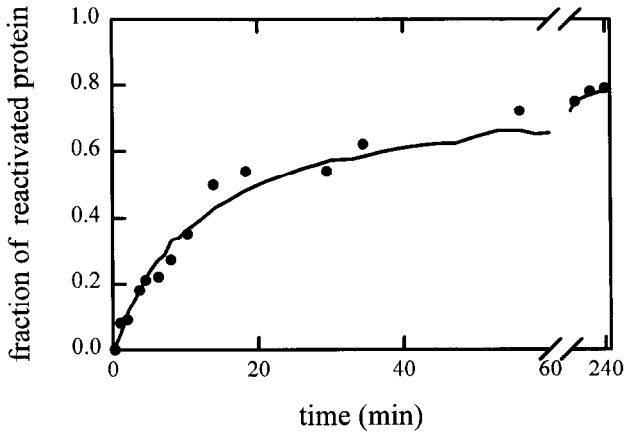

Fig. 1. Comparison of discontinuous versus continuous reactivation assay for HCAII. 17 μM HCAII was denatured in 5 M GdmCl, 0.1 M Tris/SO_4, pH 7.5 for 1 h at 20°C. Reactivation was started by rapid 17-fold dilution with 0.1 M Tris/SO_4, pH 7.5, 20°C. Reactivation determined by continuous (—) and discontinuous (●) assay method.

The CO_2 hydration activity as used by Freskgård et al. [9] can be measured by a discontinuous colorimetric method [16] where a change in colour of an indicator is determined by visual inspection.

The esterase activity of HCAII can be employed to follow reactivation by a continuous photometric assay, which uses 4-nitrophenyl acetate (4-NPA) as a substrate. This method has two advantages. The increase in product concentration can be measured directly and quantitatively by the absorbance of 4-nitrophenol at 348 nm, and the time course of HCAII-reactivation can be followed both continuously and discontinuously. We developed a continuous assay to measure the formation of native HCAII during refolding in the presence and in the absence of PPIases. To examine whether the presence of the substrate or the products influences the measured reactivation kinetics of HCAII, we compared the continuous method with a discontinuous procedure where folding occurs in the absence of the substrate. Both methods gave identical time courses for the reactivation of HCAII (Fig. 1). An influence of substrate or products, which are present in the continuous method, on the reactivation kinetics could not be detected. The results in Fig. 1 show clearly that in the absence of folding catalysts, reactivation of HCAII is not complete after 1h, as assumed previously [9]. A further increase in the amount of active protein from 64% at 1h to a final yield of 78% at 4h is observed.

3.2. Reactivation kinetics of HCAII in the absence and presence of PPIases

To reexamine the effect of different PPIases on HCAII

folding with the continuous method over an extended time, we measured the kinetics and the final yields of HCAII-reactivation in the presence of 0–20 μM rhCyp18cy and of 20 μM rhFKBP12cy (Fig.2A). In order to evaluate the influence of PPIases on the folding kinetics of HCAII, a kinetic analysis of the reactivation of HCAII is required. Reactivation in the absence of PPIases is well described by the sum of two first-order reactions with time constants of $k_1 = 0.13 \pm 0.01$ min^{-1} and $k_2 = 0.03 \pm 0.02$ min^{-1} and relative amplitudes of $A_1 = 34 \pm 3\%$ and $A_2 = 66 \pm 5\%$. The refolding of bovine carbonic anhydrase is also biphasic, and both the slow and the superslow reaction were suggested to involve prolyl isomerizations [17].

When rhCyp18cy is added at increasing concentrations both slow refolding reactions are accelerated. Maximal catalysis was observed with 20 μM rhCyp18cy and the time constants of reactivation increased to $k_1 = 0.35 \pm 0.01$ min^{-1} and $k_2 = 0.05 \pm 0.03$ min^{-1}. In addition, the relative amplitude of the faster phase increased to $A_1 = 86 \pm 5\%$ and the amplitude of the slowest phase decreased to $A_2 = 14 \pm 3\%$. A similar change in the amplitudes of refolding was observed during the catalyzed refolding of ribonuclease T1 [18]. This increase of the rates of folding coupled with the shift from the very slow to the faster phase leads to a marked acceleration of the overall reactivation process, as shown in Fig. 2. The reaction is virtually complete after 40 min, and the yield remains constant to 4 h. In the absence of PPIase, reactivation is much slower and the activity increases up to 3 h. After 4 h of reactivation identical final yields of reactivation (80 ± 3%) were observed in the presence and absence of PPIase (Fig. 2A). Inhibition of the PPIase activity by CsA results in the same reactivation kinetics and final yield as without rhCyp18cy. Porcine kidney Cyp18cy, which is 98.8% identical to rhCyp18cy [19,20], also accelerates HCAII reactivation without increasing the final yield. The yields of reactivation in the presence and absence of PPIases after 1 h (Fig. 2A) agree well with previously published results [11]. Table 1 compares the extent of reactivation after 1 h with the final yield after

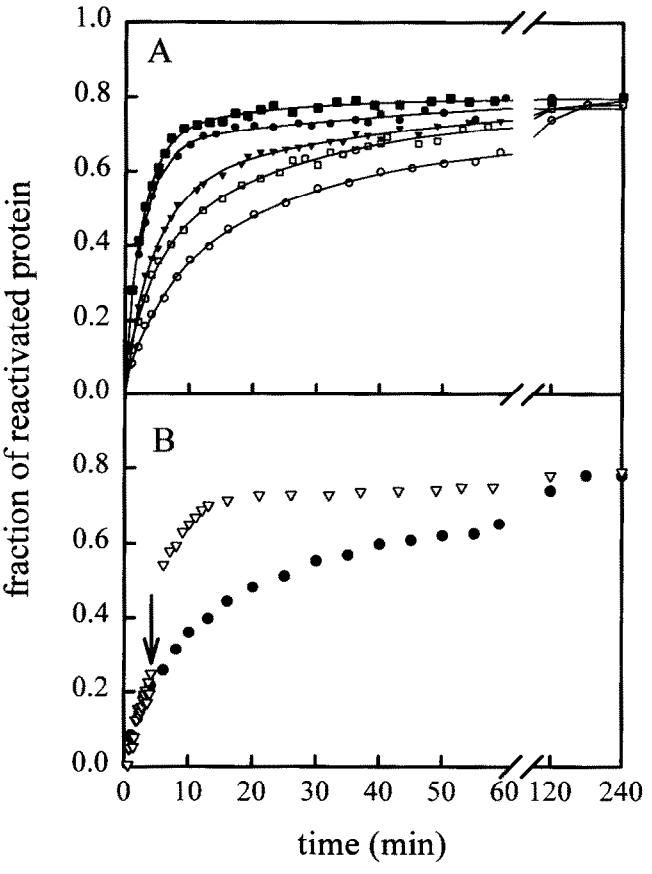

Fig. 2. A: reactivation kinetics of HCAII in the presence of rhFKBP12cy and various amounts of rhCyp18cy and in the absence of PPIases. 17 mM HCAII was denatured in 5 M GdmCl, 0.1 M Tris/SO_4, pH 7.5 for 1 h at 20°C. Reactivation was started by rapid 17-fold dilution with 0.1 M Tris/SO_4, pH 7.5, 20°C (○) in the absence of PPIases, in the presence of (□) 0.25 μM, (●) 5 μM, (■) 20 μM rhCyp18cy and (▼) 20 μM rhFKBP12cy. B: reactivation kinetics of HCAII (●) in the absence of PPIases and (▽) when 5 μM rhCyp18cy were added 4 min after the onset of reactivation (marked by arrow). Reactivation was followed with the continuous reactivation assay. Final yields were determined with both the continuous and the discontinuous assay method.

4 h in the absence and presence of different amounts of PPIases. In the presence of PPIases an increased reacti-

Table 1
Comparison of reactivation yields of long-term denatured HCAII after 1 h and 4 h in the presence of three different PPIases

PPIase		Reactivation yield for HCAII after 1 h reactivation (%)	Reactivation yield for HCAII after 4 h reactivation (%)
0 μM	rhCyp18cy	64	78
0.25 μM	rhCyp18cy	72	80
5 μM	rhCyp18cy	79	79
20 μM	rhCyp18cy	79	80
20 μM	rhFKBP12cy	71	78
10 μM	porcine kidney Cyp18cy	78	78
0 μM	porcine kidney Cyp18cy*	69	data not available
9.6 μM	porcine kidney Cyp18cy*	85	data not available

*Data determined by Franson et al. for porcine kidney Cyp18cy [11].

vation is indeed found after 1 h, but not after 4 h. Apparently, 1 h is not sufficient to reach the final reactivation value in the absence of a catalyst.

To answer the question whether other PPIases can catalyze HCAII refolding, experiments were also carried out in the presence of rhFKBP12cy. As shown in Fig. 2A this PPIase also accelerates HCAII folding, but less efficiently than rhCyp18cy. Again no increase in final yield was observed. Apparently, PPIases of both the cyclophilin and the FKBP type catalyze the slow refolding reactions of HCAII. This catalysis leads to increased extents of reactivation after intermediate times of reactivation. This effect is, however, solely due to the isomerase activity of the PPIases. After 4 h identical final reactivation yields are obtained in the absence and presence of the various PPIases.

3.3. *Double-mixing experiments*

Upon dilution of denatured HCAII into renaturation buffer an enzymatically inactive intermediate of the molten globule type is formed within less than 15 s at 20°C (see above). Chaperones are thought to bind to such early folding intermediates via hydrophobic interactions [4]. To separate a putative chaperone function from the catalytic activity of cyclophilins, we added rhCyp18cy 4 min after the reactivation of HCAII was initiated (Fig. 2B). In this experiment, cyclophilin should not be able to function as a chaperone, since the hydrophobic side chains of HCAII should be buried in the protein interior after 4 min of refolding. If Cyp18cy is a chaperone, the yield of reactivation should decrease relative to the experiment, where rhCyp18cy was present from the onset of reactivation. The delayed addition of cyclophilin leads to an acceleration of slow folding, but not to a decrease in the final yield (Fig. 2B). This result provides additional evidence that it is not necessary to assume a chaperone function to explain the effects of rhCyp18cy on the reactivation of HCAII. The experiment also shows that even after formation of the native-like structured intermediate, prolyl-*cis*/*trans* isomerization can be catalyzed by rhCyp18cy.

In conclusion, the results obtained with the improved assay method clearly show that HCAII folding is at least a biphasic process, which is not complete within 1h when PPIases are absent. After 4 h identical final yields of reactivation are observed both in the presence and absence of PPIases. The effects of cyclophilins and FKBPs on the refolding of HCAII can solely be explained by their PPIase activities, and there is no need to invoke a chaperone function for these PPIases.

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